Genetic and molecular evidence that MucA may be the sole determinant of mucoid conversion in *Pseudomonas aeruginosa*

Honors Research Thesis

Presented in Partial Fulfillment of the Requirements for graduation

"with Honors Research Distinction in Microbiology" in the undergraduate colleges of The Ohio State University

By

Kurtis Host

Project Advisor: Professor Daniel Wozniak, Department of Microbiology

Acknowledgements

I would first like to thank my mentor Dominique Limoli. The completion of this project and everything associated with it would never have been possible without her guidance. Nicki has continually set an incredible example of what an academic scientist should be; always willing to teach, never impatient and more than happy to edit anything and everything. I hope to be able to emulate her everyday hard work and persistence as I move onto my own graduate studies. I consider myself extraordinarily lucky to have worked and learned under Nicki Limoli.

Next, I would like to thank my project advisor Dr. Daniel Wozniak. The opportunity to complete this project has been awesome and has inspired me to pursue research as a career. The compassion Dan has shown me as an undergraduate is astounding. His guidance in deciding where to pursue graduate work has been indispensable. Also I would like to thank my committee Dr. John Gunn and Dr. Sheryl Justice for taking the time to evaluate this project.

The Wozniak lab has been a great environment for both growth and learning. I have felt like a part of the family from the first day and I am truly sad to leave. I will always strive to remember that there is more to life than just science. It has easily been my favorite place to work and that was crucial at points in this project. No matter how frustrating my bench work got, someone was always there to pick me up. I will forever be proud to have been a Woz lab/ski team member.

Finally, I would like to thank those closest to me, my family and friends. In particular, my wonderful girlfriend Alyssa Pace, who has helped me through all of the ups and downs of this project. She has listened to both my frantic ravings and my proud rants with a smile on her face. I could never have accomplished so much without her supporting me wholeheartedly. Her unwavering confidence has pushed me through all the times when I didn't believe in myself. My parents, Ron and Debbie Host, have given me countless words of encouragement and endless support. I am truly grateful to have been raised in such a happy, healthy home. Their constant reinforcement for doing my best has driven me to all the success I have had. I couldn't ask any more from my parents, I have been truly blessed.

The guidance my brother, Nick, has given me throughout my time at OSU has been much needed. Moreover, he has shown me what it takes to succeed both academically and professionally. My close circle of friends has been priceless for keeping me grounded through this process. I could not have accomplished this project without having them as a constant outlet.

Table of Contents

Illustrations

Abstract

Cystic Fibrosis (CF) is the most common fatal hereditary disease among Caucasians, where chronic lung infections with the bacterium *Pseudomonas aeruginosa* are the leading cause of death. In CF, *P. aeruginosa* converts to a mucoid phenotype, causing a decline in patient survival. The most common mutations responsible for mucoidy disrupt a gene encoding the anti-sigma factor MucA. MucA-independent mechanisms contributing to mucoidy have been previously proposed; however, the gene(s) responsible remain uncharacterized. As a direct result, mucoid conversion of *P. aeruginosa* remains partially unclear. The aim of this work was to identify and characterize MucA independent mucoid conversion pathways. Along the way, we found strong evidence to suggest a MucA-independent pathway may not exist. First, the *mucA* gene was sequenced from a collection of laboratory-derived mucoid isolates. 20% were found to possess a wild type *mucA* and these isolates were chosen for further investigation into a MucA independent pathway. To confirm that MucA does not control alginate overexpression on a protein level, we overproduced MucA on an inducible plasmid. However, the mucoid phenotype of all isolates was complemented upon MucA overproduction, illustrating that we have yet to isolate mucoid colonies independent of MucA. To this end, we developed a plasmid-based screen for mucoid isolates whose phenotype cannot be complemented by MucA overproduction. Surprisingly, it remained that this screen yielded only mucoid derivatives with mutations in *mucA.* Despite increasing evidence that MucA is required for mucoid conversion, the previous studies suggest a MucA independent pathway exists, which is also independent of the sigma factor AlgT/U. Therefore, to elucidate this pathway we screened for mucoid isolates in an *algT*-deficient strain. Unfortunately, four independent mucoid conversion assays with and without sublethal hydrogen peroxide treatment resulted in no mucoid isolates. Concerned by these conflicting data, we acquired the original *muc23* isolate previously published to be independent of MucA and determined that it too is complemented by MucA overexpression, demonstrating the importance of investigating post-transcriptional control of protein expression. These data suggest that MucA alterations are essential for mucoid conversion and may be the sole determinant of mucoid conversion in CF patients.

Chapter 1: Background

Pseudomonas aeruginosa lifestyle

Pseudomonas aeruginosa is an aerobic motile Gram negative rod that is naturally found in soil but is ubiquitous in most environments (1). This large range of suitable habitats is made possible because the bacterium is able to thrive in hypoxic, anaerobic and nutrient poor locations (1). The adaptability, large collection of virulence factors and recalcitrance to antibiotics of *P. aeruginosa* has made it a serious problem within hospitals. This opportunistic pathogen causes 11-14% of all nosocomial infections and 13-22% of the infections in intensive care unit patients (2). Common infection sites are the urinary tract, catheters and severe burn wounds but any immune compromised patient is at risk of infection (3). *P. aeruginosa* is especially dangerous when biofilm growth is established as it develops higher antibiotic resistant (1, 4). Virulence factors that promote infections include type IV pilus, type III secretion, exotoxin ExoU, and the pigment pyocyanin. All of these facets of *P. aeruginosa* make it very well suited for Cystic Fibrosis lung infection.

P. aeruginosa infections in Cystic Fibrosis patients

Cystic Fibrosis (CF) is the most common fatal inherited disease among Caucasians (5). Mutations present in the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) cause a defect in the mucous membrane chloride ion transport systems. Chloride ion uptake is increased without balance so the mucous produced on the lung inner surface becomes less hydrated, more viscous and more difficult for CF patients to clear. This thick mucous layer becomes a prime area for bacterial infections allowing a multitude of bacteria to establish infections including *Haemophilus influenzae* and *Staphylococcus aureus* (6). The continual colonization of the CF lung causes a chronic hyper inflammatory state predominated by neutrophils leading to increased host cell damage. This damage allows further infection and

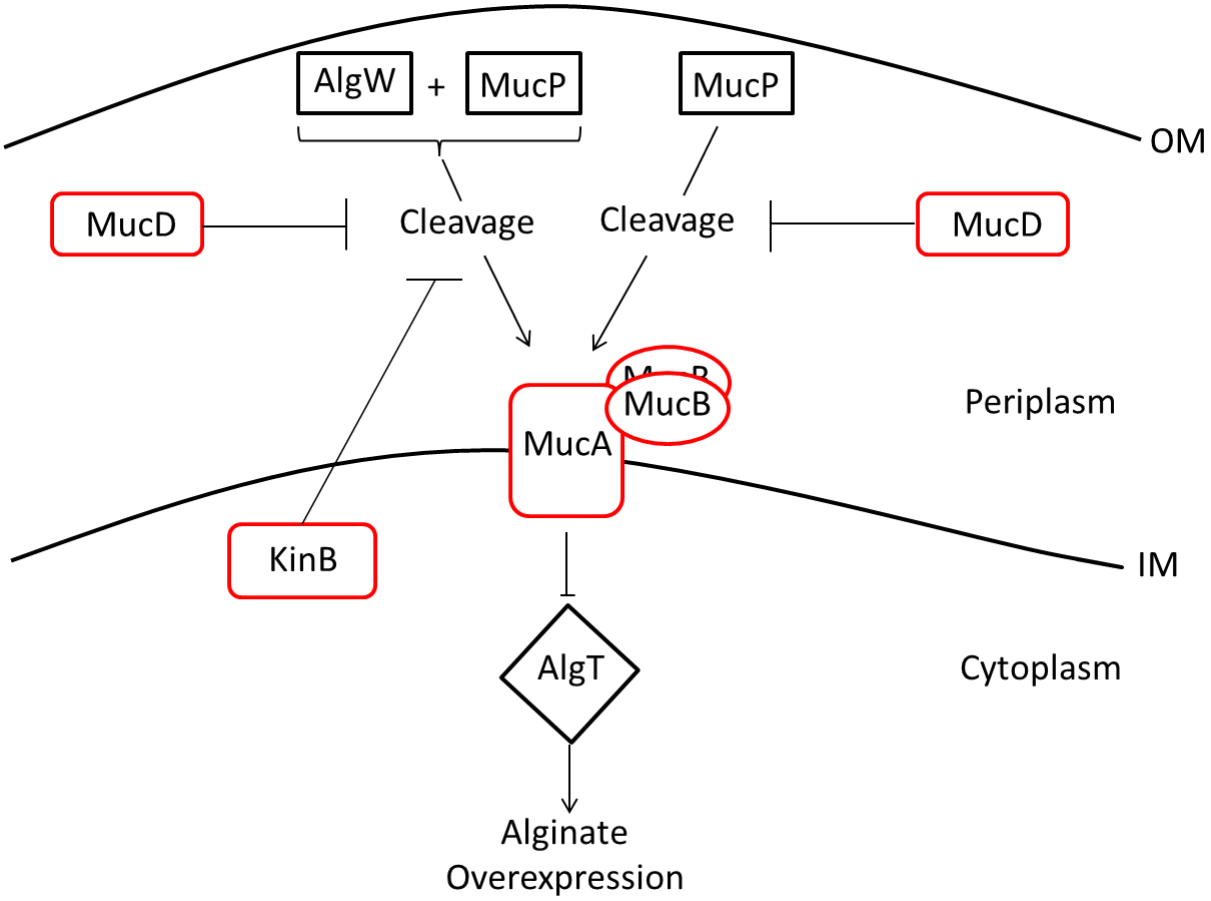
thereby perpetuates the cycle of infection to inflammation to damage. Infection with *P. aeruginosa* starts at a very young age (3yrs) but does not become the dominant infection until the teen years. Eventually leading to the death of CF patients (5). This dominance shift is correlated with a phenotypic evolution from an initial non mucoid to a mucoid phenotype. The mucoid phenotype is defined by the overproduction of the exopolysaccharide alginate. Alginate is a linear copolymer of (1-4) linked β-D-mannuronic acid and α-L-guluronic acid that acts to cover the cell in a protective coat (7). Alginate overexpression provides *P.aeruginosa* protective advantage via increased resistance to all available antibiotics and host innate immune factors thereby promoting chronic infection (8-12). Mucoid conversion is typically induced by DNA mutations resulting in a loss of regulation of alginate. Inflammatory factors which are abundant in the CF lung have been shown to induce mucoid conversion (13, 14). Moreover, mucoid *P. aeruginosa* are selected for due to increased resistance. It remains crucial to complete our understanding of mucoid conversion to be able to develop therapeutics against chronic *P. aeruginosa* infections.

Alginate Regulation

Alginate overexpression is activated by the sigma factor encoded by *algT/U* (σ^{22}). σ^{22} modulates the production of three proteins (AlgB, AmrZ, and AlgR) that allow expression of the alginate biosynthetic operon starting with the *algD* gene. AlgT is produced from a single operon containing the AlgT specific anti-sigma factor MucA (*algT-mucA-mucB-mucC-mucD*) with five promoters ranging from -452 bp to -53 bp upstream (15). MucA regulates the mucoid phenotype by binding AlgT. This 175 amino acid protein has one transmembrane domain bridging the periplasm and cytoplasm. The MucA cytoplasmic N-terminus binds σ^{22} sequestering it to the inner membrane. This MucA-AlgT interaction is stabilized by MucB binding of MucA's periplasmic C-terminus. When this MucA-AlgT interaction is destabilized alginate is overexpressed and the mucoid phenotype is established.

The most common and best characterized mechanism for mucoid conversion is *mucA* mutagenesis. These mutations render MucA nonfunctional and AlgT is no longer sequestered. Alginate is overproduced and the isolate maintains a stable mucoid phenotype. This has been found to happen in approximately 80% of clinical isolates but in 20%, a wild type (WT) *mucA* gene still remains (6,16,17). It has been previously published (17) that a WT *mucA* mucoid isolate had a stable mutation outside of the *mucA* locus termed *muc23.* The specific mutation has yet to be characterized. This knowledge is crucial if any therapeutics are to be developed. A complete picture of mucoid conversion must be understood to guarantee a safe, useful treatment against the phenotypic shift. Previous work has provided insight into the mechanism responsible for conversion in the absence of *mucA* alterations, yet the mutations responsible remain elusive (18-21). These mechanisms act post translationally via proteolysis (Fig 1) of MucA, destabilizing the MucA-AlgT interaction. The MucA protein is targeted at two sites by two separate proteases. The first protease AlgW is activated by excess amounts of MucE in the periplasm. MucD, an *E. coli* protein chaperone homologue, acts in the periplasm to suppress AlgW protease activity. Whenever *mucD* is mutated, AlgW is activated and MucA is degraded (Fig 1). Previous evidence (20) has also shown that a sensor kinase, KinB, helps to decrease AlgW activity. When mutations make KinB inactive AlgW degrades MucA (Fig 1). The second protease MucP is specific for cleavage of site 2 on MucA and is thought to follow site 1 cleavage by AlgW (19). The MucP protease alone has been shown to be sufficient to degrade MucA (19). MucP could be independently activated by unidentified stimulators in the absence of functional MucD to produce mucoid conversion. The culmination of this work has shown an intricate system in place to allow for the cleavage of MucA (Fig 1) but the nature of the *muc23* mutation and mechanism remains unclear.

Figure 1. Regulation of alginate overexpression. Schematic of regulation of MucA stability. Disruption of the red regulators (MucABD, KinB) is sufficient to cause mucoid conversion.


AlgW
+
MucP
MucP
OM
MucD
Cleavage
Cleavage
MucD
MucB
Periplasm
MucA
KinB
IM
AlgT
Cytoplasm
Alginate
Overexpression

Sigma factors and alginate overexpression

Additional insight into MucA-independent mucoid conversion mechanisms has been elucidated by investigation into alternative sigma factors able to express alginate. The *muc23* isolate has been shown to be able to overexpress alginate independent of *algT*. The RpoN alternative sigma factor σ^{54} was sufficient to maintain the mucoid phenotype when the *algT* gene was mutated (22). This suggested a novel route to mucoidy as the well-established route flows through MucA and AlgT but this strain harbored WT *mucA* and maintained mucoidy without AlgT. This strain was characterized on varying sodium and nitrogen media to view if the nitrogen response sigma factor (RpoN) would alter alginate expression. The *muc23* strain showed a differential regulation compared to *mucA* mutated isolates (23), further suggesting RpoN involvement. Therefore we sought to characterize these MucA, AlgT independent mucoid conversion mechanisms.

Summary

P. aeruginosa has proven to be a versatile bacterium and a dangerous opportunistic pathogen. This bacterium is a major concern for CF patients where *P. aeruginosa* infection is the leading cause of death (5). The shift from the non mucoid to mucoid phenotype is correlated with a severe decline in patient prognosis yet a complete picture of mucoid conversion remains elusive. MucA mutations resulting in mucoid conversion have been well characterized, but mucoidy independent of *mucA* remains elusive. Mechanisms involving post transcriptional degradation of MucA have been proposed however the specific mutations responsible are uncharacterized (18-21). The aim of this project was to identify mutations providing mucoid conversion independent of MucA. The recognition of these mutations will enhance our understanding of mucoid conversion and will be essential for development of therapeutics targeting mucoid conversion.

Chapter 2: Materials and Methods

Bacterial strains and oligonucleotides

All strains generated in this study were derived from the *Pseudomonas aeruginosa* strain PAO1*algD:cat* adapted from previous studies (24). The *algD:cat* transcriptional fusion construct was inserted into PAO1 via the nonreplicative mini-CTX vector. The plasmid DNA insertion was directed to the neutral *attB* gene. Flippase was used to excise DNA between two plasmid borne FRT regions. The removal of plasmid DNA is detected with sucrose *sacB* negative selection. Polymerase chain reaction (PCR) and sequencing analysis were used to verify *algD:cat* was correctly inserted.

The PAO1*algD:catΔalgT* strain was constructed by utilizing the pEX18Ap (25) plasmid containing the *algT* deletion construct cloned into it. Chemically competent SM10 *Escherichia cells* were used to mobilize the plasmid into PAO1*algD:cat*. Plasmid DNA was integrated into the PAO1*algD:cat* genome and directed into the *algT* gene. Sucrose *sacB* negative selection was utilized to remove the plasmid insertion and the genomic *algT*. The plasmid DNA loss will include genomic *algT* if the 500 bases upstream and downstream homologously recombine and excise the DNA in between. The resulting PAO1*algD:catΔalgT* was confirmed through PCR amplification and sequencing analysis.

Table 1

Primers	Sequence
mucA upF	5'-TGTTGCGGGATGAGATCGAGG-3'
mucA dnR	5'-GGGTGGAGAAGCTGCCATTG-3'
mucA 1F-21	5'-GGATCTTCCGCGCTCGTGAAG-3'

Table 1. Primers All primers used in this study.

Media and chemicals

Pseudomonas aeruginosa was grown on 1/2X *Pseudomonas* Isolation Agar (PIA) at 1.5% agar, Luria-Bertani Agar No Salt (LANS) (10g liter^{-1} tryptone and 5g liter^{-1} yeast extract) at 1.5% agar and Luria-Bertani No Salt (LBNS) liquid culture (10g liter^{-1} tryptone and 5g liter^{-1} yeast extract). *Escherichia coli* was grown on Luria-Bertani Agar (LA) (10g liter^{-1} tryptone, 5g liter^{-1} yeast extract and 5g liter^{-1} sodium chloride) at 1.5% agar and Luria-Bertani (LB) liquid culture (10g liter^{-1} tryptone, 5g liter^{-1} yeast extract and 5g liter^{-1} sodium chloride). All cultures were incubated at 37^{0}C for optimal growth. Antibiotic concentrations (when needed) for *P. aeruginosa* were 100 µg/ml tetracycline, 300 µg/ml carbenicillin, 250 µg/ml or 50 µg/ml chloramphenicol, 25 µg/ml irgasan, and 1 mM H_20_2. Antibiotic concentrations (when needed) for *E. coli* were 15 µg/ml tetracycline, 100 µg/ml carbenicillin, 25 µg/ml irgasan. Sucrose *sacB* negative selection against the *sacB* gene was carried out with LANS- 5% sucrose incubated at 30^{0}C.

Mucoid conversion assay

The mucoid conversion assay was developed in the laboratory by Andrea Rockel. PAO1*algD:cat* was grown overnight in 2ml LBNS. A 0.5 mL aliquot of this overnight was then

subcultured into 50ml LBNS and grown to OD 0.5. Aliquots of 5mls of culture were centrifuged at 30,000 x g and resuspended in PBS with or with H_20_2. The aliquots were incubated at 37^0C for 1 hour and then centrifuged at 30,000 x g. The pellets were resuspended in 5 ml LBNS and grown at 37^0C overnight. Aliquots of 4ml of the overnights were then centrifuged at 30,000 x g and concentrated in 1ml LBNS. This concentration was then plated onto 151.0 cm^2 chloramphenicol plates. The plates were incubated for 48 hrs at 37^0C to select for mucoid colonies. All mucoid isolates had *mucA* PCR amplified by mucA upF and mucA dnR primers at least twice. The PCR product alongside two forward primers (mucA upF, mucA 1F-21) and one reverse primer (mucA dnR) was submitted to the OSU Comprehensive Cancer Center for sequencing analysis. All *mucA* sequences were derived from the published PAO1 *mucA* sequence (26).

Complementation analysis

The generated mucoid PAO1*algD:cat* isolates had each pHERD20T and pHERD20T-*mucA* transformed separately (27). Chemically competent *E. coli* SM10 cells were used to mate plasmids into collected mucoid isolates (25). The resulting pHERD20T and pHERD20T-*mucA* containing mucoid isolates were patched from single colonies onto PIA then PIA-0.5% arabinose and PIA-5% arabinose. The plates were incubated overnight and mucoid phenotype was evaluated visually.

Chapter 3: Results

Selection strategy reveals mucoid isolates are dependent on MucA regardless of *mucA* genotype

To effectively study the mucoid conversion mechanism in *P. aeruginosa*, a large collection of mucoid isolates was needed. As the spontaneous mutation rate of *P. aeruginosa* from a nonmucoid to a mucoid phenotype is very low (10^{-8}), a selection strategy was needed. Andrea Rockel, a former graduate student in the Wozniak lab, developed an antibiotic selection in the *P. aeruginosa* strain PAO1. The *algD* promoter, which is overexpressed specifically in mucoid isolates, was transcriptionally fused to the promoterless gene for chloramphenicol acetyl-transferase (*cat*). This *algD:cat* construct allows the preferential expression of chloramphenicol resistance in mucoid isolates thereby creating antibiotic selection.

This selection strategy was utilized to generate a collection of mucoid isolates. It has been previously found that mucoid conversion is increased in the presence of human inflammatory factors (28). Therefore, to verify the utility of our selection strategy the frequency of mucoid conversion in the presence of sublethal H_2O_2 was examined compared to spontaneous mucoid conversion. The number of mucoid colonies on chloramphenicol was divided by the total colony forming units (CFUs) to determine the mucoid conversion frequency. Treatment with hydrogen peroxide increased the mucoid conversion frequency seven fold compared to baseline non-treatment conversion frequency (data not shown). A large collection of mucoid isolates have been generated using this approach and upon PCR amplification and sequencing analysis of *mucA* has shown generation of 20% of isolates with wild type *mucA* (Fig 3A). This supports the validity of the selection as it has been well established that 20% of clinical mucoid isolates maintain a wild type (WT) *mucA* (17,29).

The end goal of this project was to apply whole genome sequencing methodologies to a group of WT *mucA* mucoid isolates to genetically define alternative mucoid conversion pathways. With this in mind, we utilized pHERD20T-*mucA* to make PAO1*algD:cat* diploid for *mucA* with the reasoning that this would allow for identification of mutations in genes independent of *mucA* (Fig 4A). Unexpectedly, no WT *mucA* mucoid isolates were recovered (Fig 4B).This screen yielded mucoid derivatives with independent *mucA* mutations in both the genome and plasmid *mucA* allele (Fig 4C). The screen selected against any strains affected by WT MucA overproduction and unexpectedly both mutated *mucA* and WT *mucA* isolates reverted.

AlgT is essential for *P. aeruginosa* mucoid conversion

Previous findings (22) in a well characterized WT *mucA* mucoid isolate with a stable mutation outside of the *mucA* locus (*muc23* mutation) have shown that expression of the mucoid phenotype can occur independently of σ^{22} (encoded by *algT/U*) through the alternative sigma factor σ^{54} (RpoN). We reasoned that these σ^{54} pathways were potentially MucA independent and could be generated by forcing alginate overexpression by σ^{54}. With this in mind we generated a nonpolar, unmarked deletion of the *algT/U* gene in PAO1*algD:cat* via allelic exchange. PAO1*algD:catΔalgT* was subjected to four separate mucoid conversion assays with and without nonlethal hydrogen peroxide treatment (Fig 5). No mucoid colonies were ever recovered suggesting that the gene *algT* is essential for mucoid conversion.

The *muc23* mutation shows dependency on MucA expression

As all of our screens have failed to identify any MucA independent strains and *algT* proved to be essential for mucoid conversion, the original *muc23* strain was obtained from Dr. John Govan for characterization. This strain was subjected to the complementation assay and the mucoid

phenotype reverted upon MucA overproduction (Fig 6). This suggests that this dependency on MucA concentration is not unique to our collected strains. Even with a previously published alternative route through σ^{54} the *muc23* mutant strain remained dependent upon MucA.

All mucoid isolates revert upon MucA overexpression

In order to verify our MucA dependency findings, the original mucoid conversion assay was utilized to build a larger mucoid isolate collection to subject to pHERD20T-*mucA* complementation analysis. A total of 48 mucoid isolates have shown complementation upon MucA overproduction regardless of genotype (Fig 3B). This result reaffirms the previous findings of mucoid conversion being determined by MucA.

Sequencing of *mucA* shows a wide variety of mutations capable of rendering MucA nonfunctional

Each mucoid isolate generated was subjected to PCR and sequencing analysis of *mucA* to show genotype. This analysis has revealed a large variety of mutations within the gene (Fig 7). Each isolate has had the *mucA* gene amplified by PCR and sequenced analyzed at least twice to confirm results. The most substantial group of mutations (27%) were large (>5bp) deletions centered in *mucA*. The majority of these deletions (77%) lead to a predicted early truncation of MucA and thus produced a nonfunctional protein. This was a general trend in that only 15% of the total mutations present in *mucA* could have potentially allowed the protein to be functional. The percent of WT *mucA* isolates only represents 10% which is less than half of what is expected (20%). This number is low potentially because half of the isolates were collected in the MucA independent screen (Fig 3) that effectively selected against WT *mucA* isolates. One notable mutation missing from the list is *mucA22*. This is a guanine deletion at 426 in *mucA* and it is the most common mutation (25%) found in isolates from CF patients (30,31). With this is mind, the 4% of isolates with a guanine deletion seems a surprisingly small group. This may

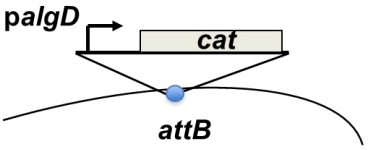
suggest that there is additional pressure within the CF lung that favors *mucA22* mutations that was not present *in vitro*. The array of mutations was wide and varied from published mutations (32). The difficulty in obtaining WT *mucA* and the wide variety of mutations to eliminate *mucA* functionality show the importance of MucA in the mucoid conversion process.

MucA may be the sole determinant of mucoid conversion

A total of 5 WT *mucA* strains have been generated, a successful antibiotic selection strategy has been developed and a large collection of mucoid isolates has been collected. An efficient screen for WT *mucA* mucoid isolates remains elusive. The goal of whole genome sequencing has not been reached as a large enough collection is yet to be gathered but a key portion of mucoid conversion has been revealed nonetheless. The initial screen to efficiently generate WT *mucA* mucoid isolates revealed unexpected results that were further tested to show that all mucoid isolates we have tested are dependent on MucA. Even the deletion of *algT* could not force any conversion mechanisms outside of *mucA* as previously proposed (22). The validation of our complementation findings with the well characterized *muc23* containing strain PAO579 shows that MucA dependency is not solely generated in our PAO1*algD:cat* but exists in independent isolates. The data gathered suggest that mucoid conversion may flow solely through MucA.

Figure 2. Selection strategy to isolate mucoid colonies **A.** Schematic of PAO1*algD:cat* construct. **B.** Flow-chart illustrating the general scheme for isolating mucoid colonies. **C.** Representative input non-mucoid and output mucoid PAO1*algD:cat* strains.

A.



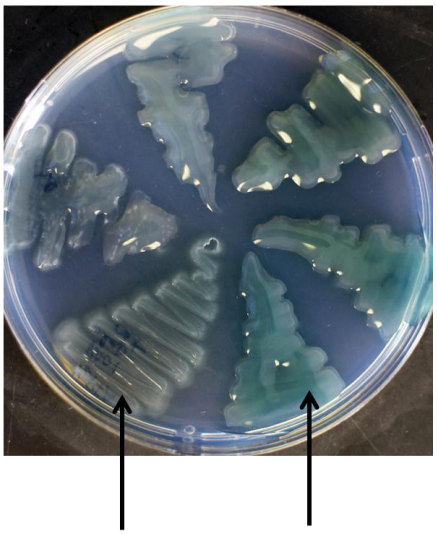
B.

Grow non-mucoid PAO1*algD::cat* for 12 hours

Plate on chloramphenicol

Recover mucoid colonies

C.



Input non-mucoid PA01*algD::cat*

Output mucoid PA01*algD::cat*

Figure 3. Sequencing and complementation analysis of *mucA* reveal mucoid isolates depend on MucA **A.** The *mucA* gene of mucoid isolates obtained via the selection strategy described in Fig. 2 was PCR amplified and the sequence determined. The percentage of isolates analyzed with *mucA* mutations (Δ*mucA*) or without (WT *mucA*) are indicated. **B.** MucA was overexpressed on a plasmid under control of an arabinose inducible promoter. Representative images are shown of the alginate phenotype when plated on *Pseudomonas* Isolation Agar containing 300 ug/ml Carbenicillin + 0.5% arabinose.

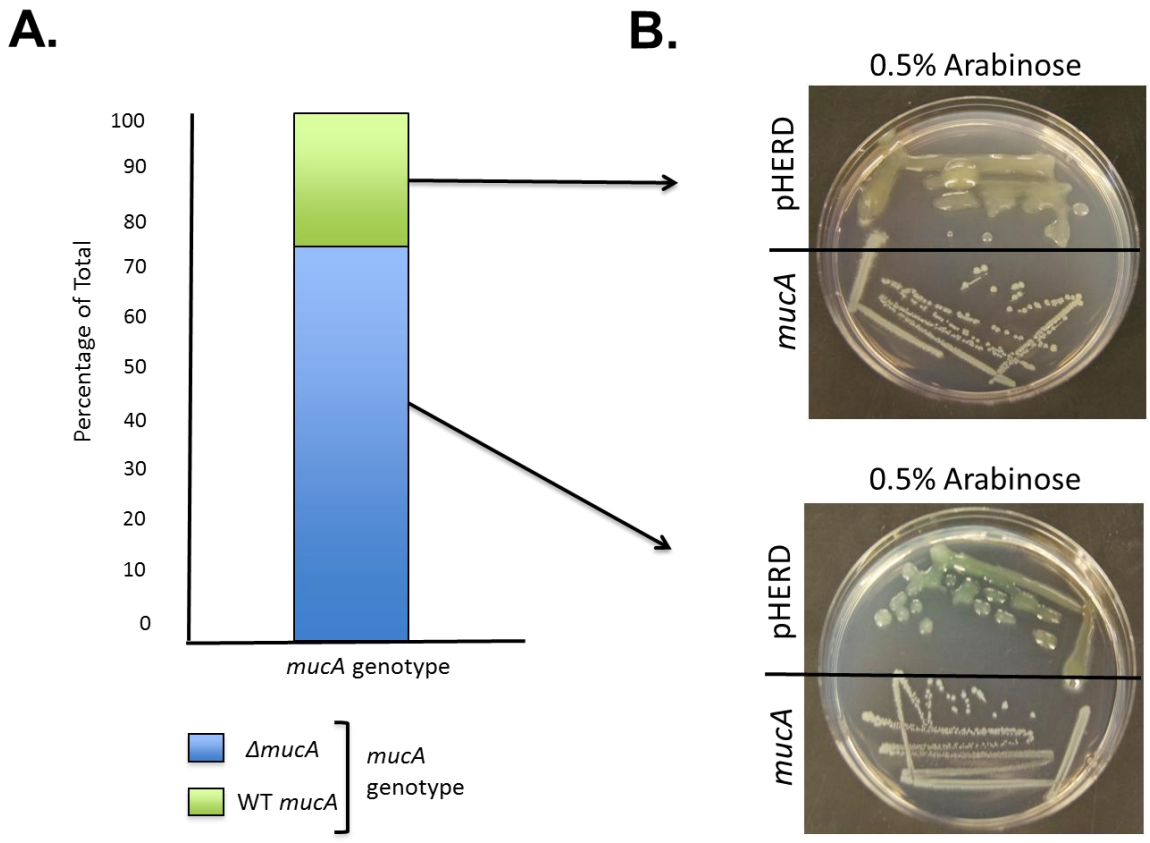

A.
B.
100
90
80
70
60
50
40
30
20
10
0
Percentage of Total
mucA genotype
ΔmucA
WT mucA
mucA genotype
0.5% Arabinose
pHERD
mucA
0.5% Arabinose
pHERD
mucA

Figure 4. Complementation-based screening for MucA-independent mucoid colonies produces solely *mucA* mutations **A.** Schematic illustrating the overall strategy to identify MucA-independent (MucA+) mucoid colonies. **B.** Results of MucA-independent screen where the alginate phenotype on *Pseudomonas* Isolation Agar (PIA) containing 300 ug/ml Carbenicillin (Cb) + 0.5% arabinose are indicated as the percent mucoid (MucA+) and non-mucoid (MucA-) of the total isolates screened (n=25). **C.** The plasmid from PAO1*algD::cat*pHERD*mucA* post-screen was isolated to examine its functionality. pHERD*mucA* was transformed into PDO300 and grown on PIA + Cb + 0.5% arabinose, followed by PCR and sequence analysis of *mucA* on the plasmid.

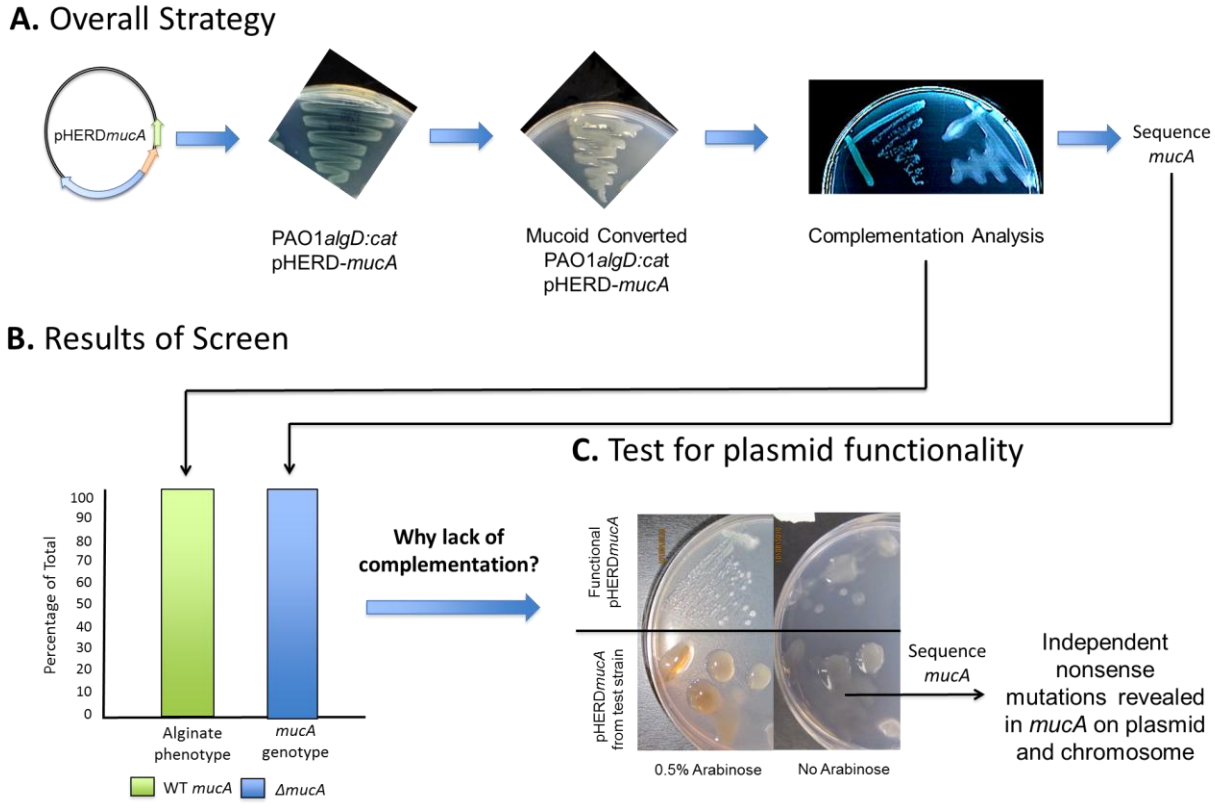

A. Overall Strategy
pHERDmucA
PAO1algD:cat
pHERD-mucA
Mucoid Converted
PAO1algD:cat
pHERD-mucA
Complementation Analysis
Sequence
mucA
B. Results of Screen
Percentage of Total
100
90
80
70
60
50
40
30
20
10
0
Alginate
phenotype
mucA
genotype
WT mucA
ΔmucA
Why lack of
complementation?
C. Test for plasmid functionality
Functional
pHERDmucA
pHERDmucA
from test strain
0.5% Arabinose
No Arabinose
Sequence
mucA
Independent
nonsense
mutations revealed
in mucA on plasmid
and chromosome

Figure 5. Mucoid colonies could not be recovered in an *algU/T*-deficient background WT PAO1*algD::cat* and PAO1*algD::catΔalgU* were grown for 12 h +/- 1.0 mM H_2O_2 and plated on 250 µg/ml chloramphenical (Cm) to enumerate the mucoid colony forming units (CFU) and on non-selective media to enumerate the total CFU. The mucoid CFU were divided by the total CFU to determine the mucoid conversion frequency. Experiments were performed in triplicate on four independent occasions and mucoid colonies were never observed in the *algU*-deficent strain. *Indicate a statistically significant difference ($P \leq 0.05$) relative to PAO1*algD::cat* + PBS using a student's *t*-test.

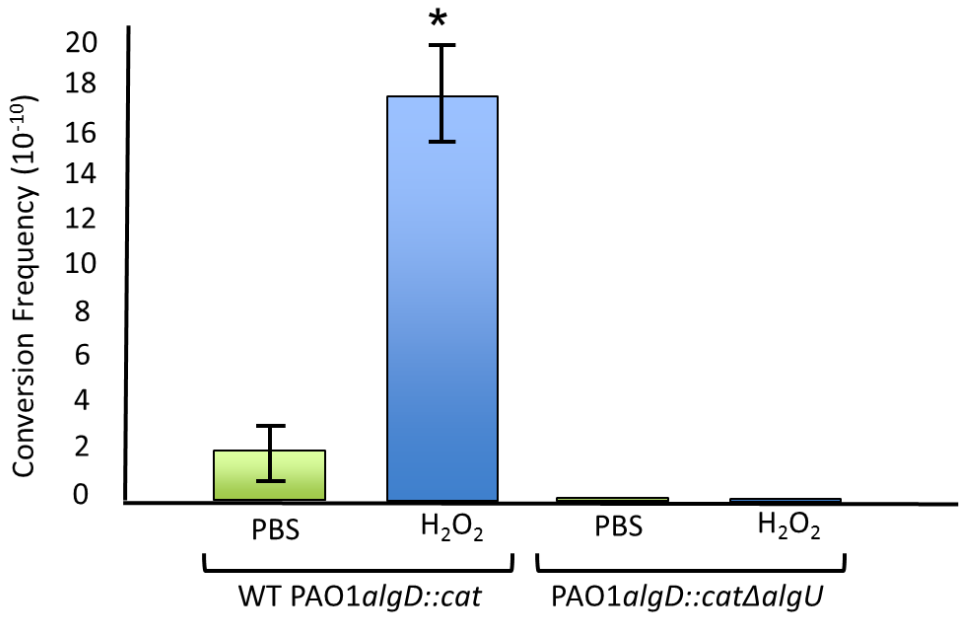

Conversion Frequency (10^{-10})
20
18
16
14
12
10
8
6
4
2
0
*
PBS
H_2O_2
PBS
H_2O_2
WT PAO1*algD::cat*
PAO1*algD::catΔalgU*

Figure 6. Isolates previously suggested to be *mucA*-independent are complemented by MucA overexpression MucA was overexpressed on a plasmid under control of an arabinose inducible promoter. Representative images are shown of the alginate phenotype when isolates are plated on *Pseudomonas* Isolation Agar containing 300 ug/ml Carbenicillin + 0.5% arabinose

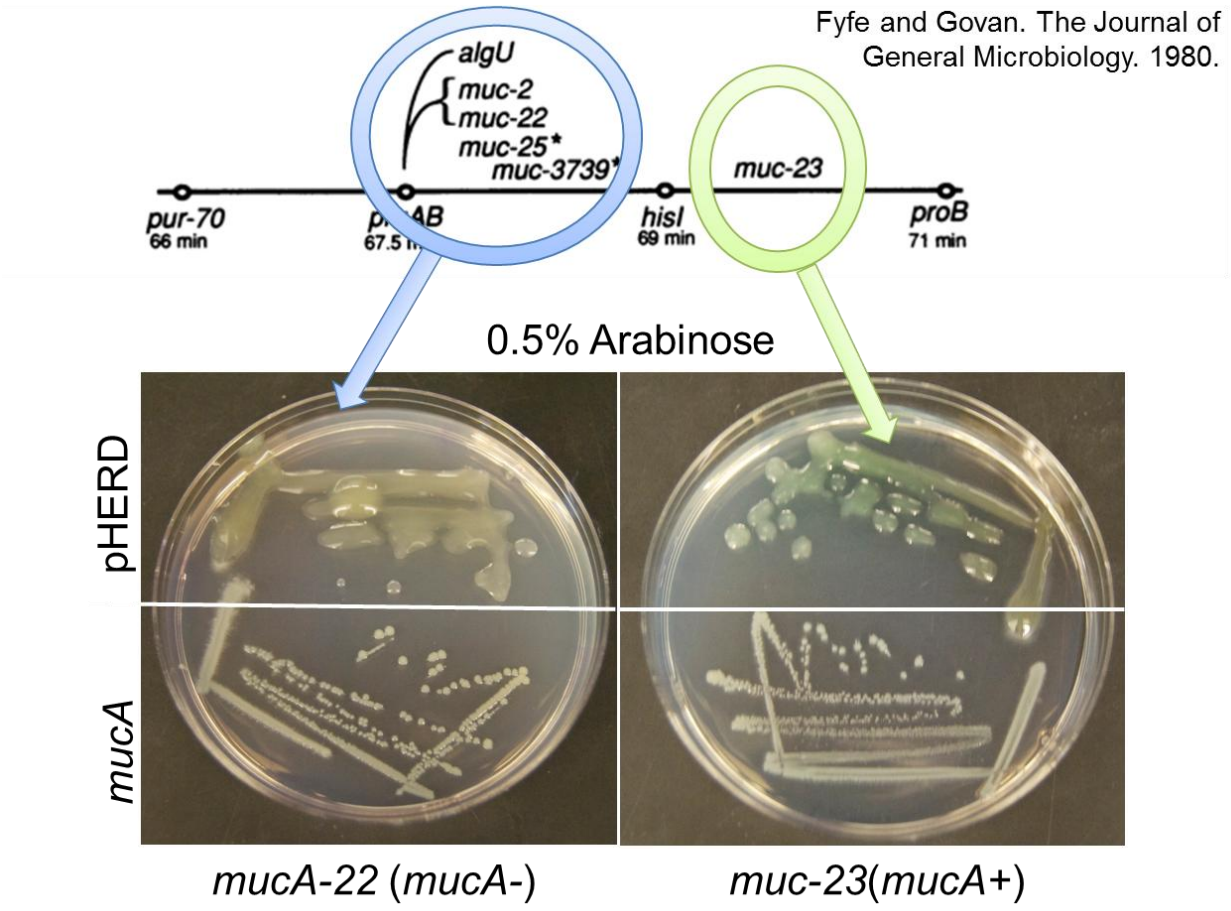
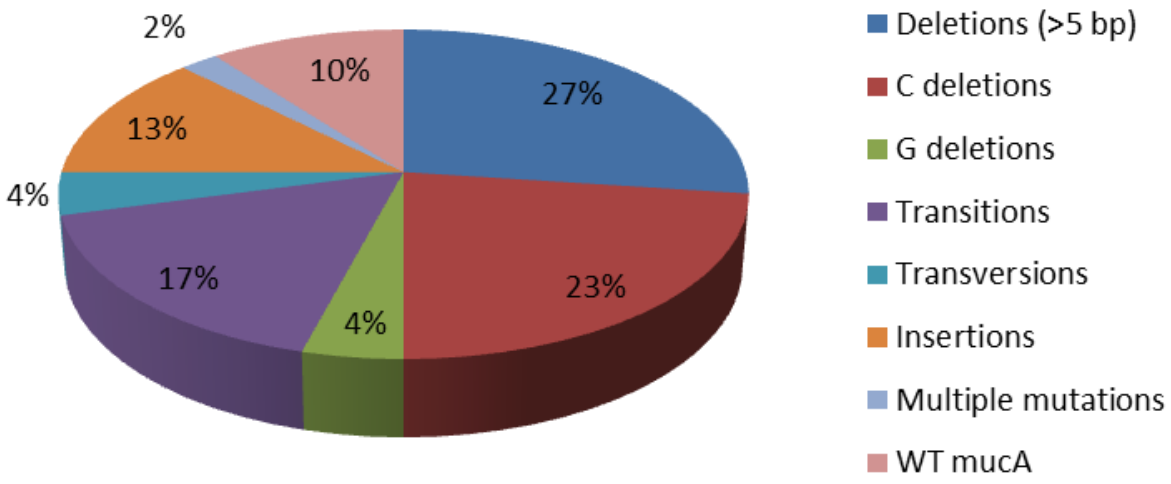

Fyfe and Govan. The Journal of General Microbiology. 1980.
algU
muc-2
muc-22
muc-25*
muc-3739*
muc-23
pur-70
66 min
hisI
69 min
proB
71 min
0.5% Arabinose
pHERD
mucA
mucA-22 (mucA-)
muc-23(mucA+)

Figure 7. Sequencing of *mucA* shows a wide variety of mutations capable of rendering MucA nonfunctional Complete collection of genotypic analysis of stable mucoid isolates used for this project. Each *mucA* gene has been PCR amplified and sequenced at least twice to ensure results. n=48


2%
10%
27%
13%
4%
17%
23%
4%
Deletions (>5 bp)
C deletions
G deletions
Transitions
Transversions
Insertions
Multiple mutations
WT mucA

Chapter 4: Discussion

The leading cause of mortality in Cystic Fibrosis patients is chronic infection with *Pseudomonas aeruginosa* (5). The shift to mucoidy is correlated directly with a severe drop in patient prognosis yet mucoid conversion mechanisms remain partially unclear. The goal of this project was to better understand mucoid conversion by defining novel mechanisms outside of *mucA* mutations. It has been previously published that stable mutations outside of the *mucA* locus (*muc23*) can produce mucoid isolates (17). With this in mind the aim of the project was to employ whole genome sequencing methods of WT *mucA* mucoid isolates that were independent of MucA. Along the way, four lines of strong evidence were gathered that suggested mucoid conversion is solely dependent on MucA. First, all isolates gathered were complemented upon MucA overexpression. Second, a screen to find MucA independent mucoid isolates failed to generate even one. Third, deletion of *algT* has shown it to be essential for mucoid conversion as no mucoid PAO1*algD:catΔalgT* isolates were recovered from the mucoid conversion assay. Fourth and finally, complementation analysis of the *muc23* isolate has shown MucA dependent reversion of mucoidy. All of the evidence gathered in this study has suggested that MucA alteration may account for all mucoid conversion events.

The future directions to find the mucoid conversion mechanisms of WT *mucA* mucoid isolates lie within elucidation of MucA alteration. There have been multiple previously published (18-21) mechanisms outside of *mucA* mutations to cause mucoid conversion. The other mechanisms primarily revolve around mutations that activate proteases (AlgW, MucP) to degrade MucA and release alginate overexpression. To view if these mutations are present in the WT *mucA* mucoid isolates, the *kinB* and *mucD* genes could be PCR amplified and sequence analyzed. Directed laboratory mutations in either gene have been demonstrated to be sufficient to cause mucoid conversion; however, it has yet to be determined if these mutations occur in CF patients. (18-21). Mutations within *kinB* are thought to cause a derepression of periplasmic

activators of AlgW. Nonfunctional MucD allows the buildup of periplasmic factors that can either induce both AlgW and MucP or just MucP proteolysis independently (19). More directly we could knockout either the AlgW or MucP proteases in our WT *mucA* mucoid isolates to determine their involvement in MucA stability. We have uncovered that the mucoid phenotype of each of these WT *mucA* isolates relies on the cellular concentration of MucA. To further confirm these finding we can analyze the amount of MucA present, the amount of MucA transcribed and translated, and visualize MucA to view potential degradation. It still remains that the group of WT *mucA* isolates could be applied to whole genome sequencing analysis to give a global view of all mutations present. Regardless of the mechanism, we have shown evidence that mucoid conversion must flow through MucA alteration. This implies that future studies may now fully focus on the MucA protein to understand any mucoid isolate.

This project has generated a lot of critical findings but much remains to be finished. The biggest step yet to be taken is analysis of clinical strains. It needs to be determined whether mucoid conversion is MucA dependent inside the CF lung. We have analyzed 30 mucoid isolates from CF patients, which we have determined all are dependent upon MucA. A reliable animal model may also provide useful to generate WT *mucA* mucoid isolate collection but one does not currently exist. Utilizing selective pressures such as inflammatory factors abundantly present in the CF lung could help in generating more clinically relevant mutations. No treatment was used in the conversion assays in an effort to reduce background mutations in the gathered WT *mucA* mucoid isolates to allow for easier whole genomic analysis.

The evidence gathered in this project has relied heavily on the overproduction of MucA from the pHERD20T-*mucA* plasmid. The levels of MucA being produced will need to be measured to ensure that our complementation results have not been generated as a result of MucA protein overwhelming any other mechanism present. We have attempted to address this caveat by performing our complementation assay on minimal inducing arabinose (0.5%) and

ten-fold higher (5%) as pHERD20T-*mucA* MucA production is arabinose dose dependent. The two arabinose concentrations did not show any differences. This concern could be addressed in the future by utilizing a lower copy plasmid and finding the lowest production of MucA to result in complementation. If MucA overproduction is found to be overwhelming the mechanisms present in the WT *mucA* mucoid isolates then MucA independent pathways may yet be identified.

Another potential caution is that we could not produce a mucoid isolate that lacked *algT* whereas the *muc23* strain has been published to remain mucoid upon *algT* mutagenesis (22). The *algT* gene was disrupted but not completely removed and mutagenesis came after mucoid conversion. We have attempted to make a nonpolar, unmarked *algT* deletion in the *muc23* strain but we were unsuccessful on numerous attempts. It may be that some portion of AlgT needs to remain intact in the cell to allow the *muc23* mutation to be nonlethal. This is understandable when it is noted that σ^{22} is essential in *E. coli* (33). Also, technically this deletion is difficult to achieve as it is a rare double recombination event with a weak negative selection. Further analysis will need to be done to understand this necessity for *algT* in both maintenance of and conversion to the mucoid phenotype.

The sequencing analysis performed uncovered the fact that the mutations present in *mucA* do not contain *mucA22*. This is troubling as this is the most frequent *mucA* mutation identified in clinical isolates from CF patients (30,31). The conversion assay may need to be altered to better reflect CF lung selective pressures to gather more clinically relevant mutations.

The evidence gathered thus far has allowed us to shed more light on the phenotypic shift that is correlated with chronic infection. We have identified the single protein that must be altered in order to allow the mucoid phenotype and identified a sigma factor essential for conversion to the phenotype. These findings alongside previous proteolysis studies have helped to more completely elucidate the mechanism for mucoid conversion outside of *mucA* mutations.

The initial goal of this study was to find any mucoid mechanism independent of *mucA* in the thought that if treatment were developed for *mucA* dependent mechanisms it would only divert down a separate pathway. This study has found no other pathway suggesting that therapeutics targeted at MucA will not generate mucoid conversion through separate pathways. MucA may now be a useful and more importantly safe target for therapeutics. This work, reinforcing post translational alterations of MucA, has brought us closer to a complete understanding of mucoid conversion and a great potential target for therapeutics.

References

1. Hancock REW, Speert DP. Antibiotic resistance in Pseudomonas aeruginosa: mechanisms and impact on treatment. *Drug Resistance Updates*. 2000;3(4):247-255. Available at: http://linkinghub.elsevier.com/retrieve/pii/S1368764600901523.

2. Hidron AI, Edwards JR, Patel J, et al. NHSN annual update: antimicrobial-resistant pathogens associated with healthcare-associated infections: annual summary of data reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2006-2007. *Infection control and hospital epidemiology : the official journal of the Society of Hospital Epidemiologists of America*. 2008;29(11). Available at: http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=18947320&retmode=ref&cmd=prlinks.

3. Hogardt M, Heesemann J. Adaptation of Pseudomonas aeruginosa during persistence in the cystic fibrosis lung. *International Journal of Medical Microbiology*. 2010;300(8):557-562. Available at: http://linkinghub.elsevier.com/retrieve/pii/S1438422110000883.

4. Donlan RM, Costerton JW. Biofilms: Survival Mechanisms of Clinically Relevant Microorganisms. *Clinical Microbiology Reviews*. 2002;15(2):167-193. Available at: http://cmr.asm.org/cgi/doi/10.1128/CMR.15.2.167-193.2002.

5. Doull I. Recent advances in cystic fibrosis. *Archives of disease in childhood*. 2001. Available at: http://adc.bmj.com/content/85/1/62.abstract.

6. Govan JR, Deretic V. Microbial pathogenesis in cystic fibrosis: mucoid Pseudomonas aeruginosa and Burkholderia cepacia. *Microbiological Reviews*. 1996;60(3). Available at: http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=8840786&retmode=ref&cmd=prlinks.

7. Ryder C, Byrd M, Wozniak DJ. Role of polysaccharides in Pseudomonas aeruginosa biofilm development. *Current Opinion in Microbiology*. 2007;10(6):644-648. Available at: http://linkinghub.elsevier.com/retrieve/pii/S136952740700135X.

8. Schwarzmann S. Antiphagocytic effect of slime from a mucoid strain of Pseudomonas aeruginosa. *Infection and Immunity*. 1971. Available at: http://iai.asm.org/cgi/content/abstract/3/6/762.

9. Ryder C, Byrd M, Wozniak DJ. Role of polysaccharides in Pseudomonas aeruginosa biofilm development. *Current Opinion in Microbiology*. 2007;10(6):644-8. Available at: http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=17981495&retmode=ref&cmd=prlinks.

10. Yormiar, Ramsey DM, Wozniak DJ. Understanding the control of Pseudomonas aeruginosa alginate synthesis and the prospects for management of chronic infections in cystic fibrosis. *Molecular Microbiology*. 2005;56(2):309-322. Available at: http://doi.wiley.com/10.1111/j.1365-2958.2005.04552.x.

11. Slack MP, Nichols WW. The penetration of antibiotics through sodium alginate and through the exopolysaccharide of a mucoid strain of Pseudomonas aeruginosa. *Lancet*. 1981;2(8245):502-3. Available at: http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=6115250&retmode=ref&cmd=prlinks.

12. Microbial pathogenesis in cystic fibrosis: mucoid Pseudomonas aeruginosa and Burkholderia cepacia. *Microbiological Reviews*. 1996. Available at: http://mmbr.asm.org/content/60/3/539.abstract.

13. Hassett D. Bacterial adaptation to oxidative stress: implications for pathogenesis and interaction with phagocytic cells. *The FASEB Journal*. 1989. Available at: http://www.fasebj.org/content/3/14/2574.short.

14. WILLIAMS BJ, DEHNBOSTEL J, BLACKWELL TS. Pseudomonas aeruginosa: Host defence in lung diseases. *Respirology*. 2010;15(7):1037-1056. Available at: http://doi.wiley.com/10.1111/j.1440-1843.2010.01819.x.

15. Schurr M, Yu H, Boucher J, Hibler N. Multiple promoters and induction by heat shock of the gene encoding the alternative sigma factor AlgU (sigma E) which controls mucoidy in cystic fibrosis isolates of &hellip. *Journal of*. 1995. Available at: http://jb.asm.org/cgi/content/abstract/177/19/5670.

16. Martin D, Schurr M, Mudd M. Mechanism of conversion to mucoidy in Pseudomonas aeruginosa infecting cystic fibrosis patients. In: *Proceedings of the*. 1993. Available at: http://www.pnas.org/content/90/18/8377.short.

17. FYFE J. Alginate synthesis in mucoid Pseudomonas aeruginosa: a chromosomal locus involved in control. *Journal of general microbiology*. 1980. Available at: http://mic.sgmjournals.org/content/119/2/443.short.

18. Damron FH, Owings JP, Okkotsu Y, et al. Analysis of the Pseudomonas aeruginosa regulon controlled by the sensor kinase KinB and sigma factor RpoN. *Journal of Bacteriology*. 2012. Available at: http://jb.asm.org/cgi/doi/10.1128/JB.06105-11.

19. Damron FH, Yu HD. Pseudomonas aeruginosa MucD Regulates the Alginate Pathway through Activation of MucA Degradation via MucP Proteolytic Activity. *Journal of Bacteriology*. 2010;193(1):286-291. Available at: http://jb.asm.org/cgi/doi/10.1128/JB.01132-10.

20. Damron FH, Qiu D, Yu HD. The Pseudomonas aeruginosa Sensor Kinase KinB Negatively Controls Alginate Production through AlgW-Dependent MucA Proteolysis. *Journal of Bacteriology*. 2009;191(7):2285-2295. Available at: http://jb.asm.org/cgi/doi/10.1128/JB.01490-08.

21. Wood L, Leech A. Cell wall‐inhibitory antibiotics activate the alginate biosynthesis operon in Pseudomonas aeruginosa: roles of σ22 (AlgT) and the AlgW and Prc

proteases. *Molecular Microbiology*. 2006. Available at: http://onlinelibrary.wiley.com/doi/10.1111/j.1365-2958.2006.05390.x/full.

22. Boucher J, Schurr M. Dual regulation of mucoidy in Pseudomonas aeruginosa and sigma factor antagonism. *Molecular Microbiology*. 2000. Available at: http://onlinelibrary.wiley.com/doi/10.1046/j.1365-2958.2000.01846.x/full.

23. Deretic von, Govan J, Konyecsni W. Mucoid Pseudomonas aeruginosa in cystic fibrosis: mutations in the muc loci affect transcription of the algR and algD genes in response to environmental stimuli. *Molecular*. 1990. Available at: http://onlinelibrary.wiley.com/doi/10.1111/j.1365-2958.1990.tb00586.x/abstract.

24. Wood LF, Leech AJ, Ohman DE. Cell wall-inhibitory antibiotics activate the alginate biosynthesis operon in Pseudomonas aeruginosa: Roles of sigma (AlgT) and the AlgW and Prc proteases. *Molecular Microbiology*. 2006;62(2):412-26. Available at: http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=17020580&retmode=ref&cmd=prlinks.

25. Hoang TT, Karkhoff-Schweizer RR, Kutchma AJ, Schweizer HP. A broad-host-range Flp-FRT recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked Pseudomonas aeruginosa mutants. *Gene*. 1998;212(1):77-86. Available at: http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=9661666&retmode=ref&cmd=prlinks.

26. Stover CK, Pham XQ, Erwin AL, et al. Complete genome sequence of Pseudomonas aeruginosa PAO1, an opportunistic pathogen. *Nature*. 2000;406(6799):959-64. Available at: http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=10984043&retmode=ref&cmd=prlinks.

27. Qiu D, Damron FH, Mima T, Schweizer HP, Yu HD. PBAD-Based Shuttle Vectors for Functional Analysis of Toxic and Highly Regulated Genes in Pseudomonas and Burkholderia spp. and Other Bacteria. *Applied and Environmental Microbiology*. 2008;74(23):7422-7426. Available at: http://aem.asm.org/cgi/doi/10.1128/AEM.01369-08.

28. Mathee K, Ciofu O, Sternberg C. Mucoid conversion of Pseudomonas aeruginos by hydrogen peroxide: a mechanism for virulence activation in the cystic fibrosis lung. *Microbiology*. 1999. Available at: http://mic.sgmjournals.org/content/145/6/1349.short.

29. Flynn J. Cloning of genes from mucoid Pseudomonas aeruginosa which control spontaneous conversion to the alginate production phenotype. *Journal of Bacteriology*. 1988. Available at: http://jb.asm.org/cgi/content/abstract/170/4/1452.

30. Martin D, Holloway B. Characterization of a locus determining the mucoid status of Pseudomonas aeruginosa: AlgU shows sequence similarities with a Bacillus sigma factor. *Journal of Bacteriology*. 1993. Available at: http://jb.asm.org/cgi/content/abstract/175/4/1153.

31. Goldberg J, Gorman W, Flynn J. A mutation in algN permits trans activation of alginate production by algT in Pseudomonas species. *Journal of*. 1993. Available at: http://jb.asm.org/cgi/content/abstract/175/5/1303.

32. Moyano AJ, Smania AM. Simple Sequence Repeats and Mucoid Conversion: Biased mucA Mutagenesis in Mismatch Repair-Deficient Pseudomonas aeruginosa Herman C, ed. *PLoS ONE*. 2009;4(12):e8203. Available at: http://dx.plos.org/10.1371/journal.pone.0008203.

33. de Peñas AL, Connolly L, Gross CA. SigmaE is an essential sigma factor in Escherichia coli. *Journal of Bacteriology*. 1997;179(21):6862-4. Available at: http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=9352942&retmode=ref&cmd=prlinks.